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# Evaluation of Reproductive Parameters in Adult Male Wistar Rats After Subchronic Exposure (Gavage) to Benomyl

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# EVALUATION OF REPRODUCTIVE PARAMETERS IN ADULT MALE WISTAR RATS AFTER SUBCHRONIC EXPOSURE (GAVAGE) TO BENOMYL

**Ralph E. Linder, Georgia L. Rehnberg**

Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina

**Lillian F. Strader, Juanita P. Diggs**

Health Effects Research, Northrop Services, Inc.—Environmental Sciences, Research Triangle Park, North Carolina

*Proven-breeder 102-d-old male Wistar rats were gavaged daily with 0, 1, 5, 15, or 45 mg/kg·d benomyl. The animals were bred to untreated females after 62 d and killed after 76–79 d for evaluation of selected male reproductive end points. Minimal to moderate changes were observed in rats dosed with 45 mg/kg·d; these included decreased testis and epididymis weight, reduced cauda sperm reserves, decreased sperm production, increased numbers of decapitated spermatozoa, and increased numbers of seminiferous tubules containing multinucleated giant cells. Reproductive performance, seminal vesicle and prostate weight, sperm motility, serum luteinizing hormone, follicle-stimulating hormone, prolactin, and androgen binding protein were not affected by any of the dosages tested. Based on these end points, the no-effect level was 15 mg/kg·d.*

## INTRODUCTION

Benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, a systemic fungicide with broad-spectrum activity against a variety of pathogenic plant fungi, is widely used on numerous food crops and ornamental plants. The fungicidal properties of benomyl are generally attributed to the antimitotic activity of its conversion product, methyl 2-

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Requests for reprints should be sent to Ralph E. Linder, Developmental and Cellular Toxicology Division, Health Effects Research Laboratory (MD 72), Research Triangle Park, North Carolina 27711.



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benzimidazolecarbamate (MBC), which binds to fungal tubulins and may interfere with the assembly of microtubules (Clemons and Sisler, 1971; Davidse, 1973; Hammerschlag and Sisler, 1973; Davidse and Flach, 1977).

Cytotoxic and cytogenetic effects of benomyl and/or MBC have been observed in in vitro and in vivo mammalian test systems (Styles and Garner, 1974). Additional studies in intact laboratory animals indicate that the mammalian testis is a primary target of benomyl. Chronic exposure (4–11 mo) to benomyl (500 mg/kg·d) administered by gavage produced testicular changes and decreased fertility in male Wistar rats (Torchinskiy et al., 1976; Ivanova-Chemishanska and Antov, 1980). Carter et al. (1984) observed testicular changes after 5 daily doses of 250 mg/kg and suggested that the adult (75-d-old) Sprague-Dawley rat may be more susceptible than the pubertal (54-d-old) and prepubertal (33-d-old) animal. Acute exposures also elicit adverse testicular effects; within 48 h, a single oral dose of 400 mg/kg resulted in massive exfoliation of spermatogenic cells and testicular edema in Sprague-Dawley rats (Hess et al., 1985). In contrast, Sherman et al. (1975) observed no effects on reproductive performance over 3 generations when Sprague-Dawley rats were fed 2500 ppm in the diet for 13 wk from weaning; the lack of toxicity in these growing rats might be due to the maximum dietary dosage (milligrams per kilograms per day) occurring during the prepubertal and pubertal stages of development. Conflicting data on the potency of dietary administration of benomyl have been presented by Barnes et al. (1983), who reported decreased testicular weight and reduced fertility in adult male Wistar rats at a dietary level of 1 ppm.

The method of dosing may influence the toxicity of benomyl. For example, Kavlock et al. (1982) found significant teratogenic effects at dosages of 62.5 mg/kg·d) when benomyl was administered by gavage to pregnant Wistar rats, whereas 505 mg/kg·d administered via the diet resulted in only mild retardation of fetal growth. As these authors discussed, differences between toxicity in dietary versus gavage treatment might be attributed to detoxification mechanisms being overwhelmed by temporally concentrating the dose as in gavage treatment. They argued that gavage treatment may be more relevant to humans who might consume contaminated food products during brief discrete time periods.

We undertook the present study utilizing a model that the literature suggested as being both relevant and sensitive, that is, the adult male Wistar rat dosed subchronically by gavage. The lower end of the anticipated dose-response range for male reproductive toxicity was investigated. Evaluations of reproductive performance, spermatogenesis (sperm production, motility, morphology, and storage), reproductive organ histology, and selected serum hormonal measurements are presented.

## METHODS

To obtain proven breeder males, 60-d-old male and female Wistar rats purchased from Charles River Breeding Laboratories, Kingston, N.Y., were allowed to acclimate to laboratory conditions for 12 d, and then pair-mated for 2 wk. Sixty males that had sired a litter of at least 9 live pups were ranked by body weight and distributed randomly into 5 groups of 12 rats each. The animals were housed 2 per cage in a class 100 laminar flow room (Bioclean) under conditions of 12 h light/12 h dark, 72 ± 2°F, and 50 ± 10% relative humidity. Purina Laboratory Chow and tap water were provided ad libitum. Benomyl of 98.6% purity (manufacturer's specifications) was obtained from E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.; confirmatory analysis of this material gave a purity value of 97.5%. A 1% suspension of the benomyl in corn oil was homogenized in a Sorvall Omni-mixer and dilutions of 0.02, 0.1, 0.3, and 0.9% were prepared from the concentrate. The suspensions were prepared each day just prior to dosing and administered by oral gavage at the rate of 5 ml/kg body weight to obtain dosages of 0 (corn oil only), 1, 5, 15, and 45 mg/kg·d. The intermediate dosages approximated the no-effect levels reported by Torchinskiy et al. (1976) and Kavlock et al. (1982), while the highest dosage was approximately 33% higher than the minimum toxic dosage reported in these studies. Dosing was started when the animals were 102 d old and dose volumes were adjusted weekly for body weight gain.

The animals were bred after 62 doses; this interval corresponds to the approximate duration of spermatogenesis plus epididymal transit time of spermatozoa in the rat (Clermont, 1972; Robb et at., 1978). Each male was caged with an untreated virgin female for a minimum of 5 d or until insemination occurred. When daily cage inspections indicated mating (vaginal plug or coagulum fragments in the cage pan), vaginal smears were examined for the presence of sperm. Sperm-positive females were separated from the males and a second female was paired with each male until inseminated. Females with confirmed insemination dates were sacrificed on d 20 of gestation, and the total number of implantation sites, the number of live fetuses, and the pooled body weights for pups of each sex were obtained for each litter. Females with unconfirmed insemination dates were also examined for live and dead implants 2 wk after separation from the males.

The treated males were sacrificed for terminal studies after 76–79 daily doses. Under ether anesthesia, blood was drawn from the abdominal aorta, and the animals were killed by exsanguination. Luminal fluid from the left or right cauda epididymis (alternating for each animal) was collected in a micropipette and suspensions were prepared for evaluation of sperm concentration and motility (Linder et al., 1986). For sperm morphology, the sperm suspensions were subsequently diluted 1:1

with 10% formalin to preserve the cells and examined by phase-contrast microscopy at 200×; for each animal, 200 spermatozoa were evaluated for head and tail abnormalities. The contralateral tests and epididymides were excised, weighed, and used to determine sonication-resistant sperm heads (Cassidy et al., 1983) and cauda sperm reserves (caudae were minced in 1 ml of warm saline, diluted approximately 1:250 with 10% formalin, and the spermatozoa were counted using a hemacytometer).

The remaining testes and epididymides, seminal vesicles, and prostates were weighed and fixed in 2% glutaraldehyde with 4% paraformaldehyde for microscopic study. Seminal vesicles and prostates were embedded in paraffin and stained with hematoxylin and eosin (H&E). The testes and epididymides from one-half the animals in each group were embedded in paraffin; tissues from the remaining animals were embedded in glycol methacrylate. Transverse sections of the testis and longitudinal sections of the caput and cauda epididymides were stained with H&E and periodic acid-Schiff (PAS) stain. Quantitative counts of the number of (1) seminiferous tubules with one or more multinucleated giant cells (MNGC), (2) atrophic tubules (Sertoli cell only or occasional germ cells), or (3) partially atrophic tubules (some germ cell loss or disorganization) were made on three testicular cross sections taken at three levels of the testis for each animal. Total tubules per cross section were determined from photographs of whole cross sections of the testis.

Serum was assayed for luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL) by radioimmunoassay (RIA) (Niswender et al., 1969) using reagents obtained from the National Hormone and Pituitary Program. Carrier-free iodine-125 was obtained from New England Nuclear (Boston, Mass). Pure rat LH (NIADDK-rLH-I-6), FSH (NIADDK-rFSH-I-3), and PRL (NIADDK-rPRL-I-5) were iodinated using the chloramine T method (Greenwood et al., 1963). The LH values are expressed in terms of NIADDK-rLH-RP-2. The sensitivity of the LH RIA was 15 pg/tube. The antiserum to LH was NIADDK-antirat-LH-S-9 at a dilution of 1:40,000. FSH values are expressed in terms of NIADDK-rFSH-RP-2. The sensitivity of the FSH RIA was 3 pg/tube. The antiserum to FSH used was NIADDK-antirat-FSH-S-11 at a dilution of 1:2500. PRL values are expressed in terms of NIADDK-rPRL-PR-3. The sensitivity of the assay was 32 pg/tube. The antiserum to PRL used was NIADDK-antirat-PRL-S-9 at a dilution of 1:12,500. Serum androgen binding protein (ABP) was determined by RIA (Gunsalas et al., 1978) using reagents supplied by the National Hormone and Pituitary Program, NICHD. Pure rat ABP was iodinated using the iodogen method (Fracker and Speck, 1978). ABP values are expressed in terms of WR-R1 and the sensitivity of the RIA was 0.2 ng/tube. Intraassay variabilities for LH, FSH, PRL, and ABP were 8.1, 12.4, 9.0, and 12.0%, respectively. Interassay variabilities for LH, FSH, PRL, and ABP were 7.7, 8.3, 1.9, and 9.5%, respectively.

Sperm counts, body weights, and hormonal measurements were log transformed and evaluated by analysis of variance and Duncan's multiple range test. Organ weights and the sperm motility counts were subjected to analysis of covariance using the final body weight and nonmotile sperm per milligram cauda fluid as the covariants; least-square means were used in the group comparisons. Wilcoxon scores and the Kruskal-Wallis test were used to evaluate litter size, pup viability, sperm morphology, and quantitative histology data. Fisher's exact test was used to compare the incidence of animals with histologic abnormalities.

## RESULTS

Benomyl treatment produced no clinical signs of toxicity or adverse effect on body weight gain. One rat treated with 1 mg/kg·d died, apparently from a dosing injury.

The reproductive performance of male rats treated with benomyl appears in Table 1. With the exception of 1 animal dosed with 1 mg/kg·d, all males sired at least 1 litter. The percentage of matings that resulted in live litters ranged from 75% in the groups dosed with 5 and 15 mg/kg·d to 100% in the group dosed with 45 mg/kg·d. No adverse effects on reproductive performance could be attributed to the benomyl dosages used.

The weights of reproductive organs appear in Table 2. Compared to the control group, a 10% and 16% decrease in testis and epididymis weight, respectively occurred in animals treated with 45 mg/kg·d, but the weights of these organs were unaffected at lower dosages. No effects on the weights of the seminal vesicles or prostate were observed.

Several sperm parameters were affected in animals dosed with 45 mg/kg·d (Table 3). The percentage of morphologically normal spermatozoa was decreased (93.8% vs. 98.1% for controls), primarily because of increased numbers of decapitated cells. The proportion of progressively motile spermatozoa (motility index) was unaffected. The number of spermatozoa in the cauda epididymis was markedly decreased (44%), although the sperm concentration in the cauda luminal fluid was unchanged. Sperm production based on the number of sonication-resistant sperm heads per testis was 87% of control values ($p > 0.05$), but this small decrease was probably treatment related; when conservative criteria ($\alpha = 0.005$) for rejection of large outlying values were applied to each group (Natrella, 1966), 1 value ($285 \times 10^6$) for sperm heads per testis could be excluded from the group dosed with 45 mg/kg·d. Exclusion of this value reduced the group mean to $193 \pm 4 \times 10^6$, compared to $230 \pm 9 \times 10^6$ for the controls ($p = 0.001$ by analysis of variance). Sperm production based on the number of sperm heads per gram of testis parenchyma was unaltered. No adverse

**TABLE 1.** Reproduction in Male Rats Treated with Benomyl

| Treatment (mg/kg·d) | Number mated | | Copulatory plugs | Sperm-positive smears | Total litters | Fertile males | Pups per litter | Pup weight | | Implantation sites per litter | Resorptions per litter (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | | | | | | M (g) | F (g) | | |
| 0 | 12 | 24 | 22 | 22 | 23 | 12 | 14.7 ± 0.9[a] | 3.9 ± 0.1 | 3.7 ± 0.1 | 15.7 ± 0.9 | 6.1 ± 1.7 |
| 1 | 11 | 22 | 21 | 20 | 19 | 10 | 14.6 ± 0.6 | 3.8 ± 0.1 | 3.6 ± 0.1 | 16.0 ± 0.6 | 9.3 ± 1.6 |
| 5 | 12 | 24 | 18 | 18 | 19 | 12 | 14.6 ± 1.0 | 3.9 ± 0.1 | 3.6 ± 0.1 | 15.8 ± 0.9 | 7.8 ± 2.0 |
| 15 | 12 | 24 | 21 | 19 | 18 | 12 | 14.6 ± 0.9 | 3.7 ± 0.1 | 3.6 ± 0.1 | 15.3 ± 0.3 | 4.4 ± 1.4 |
| 45 | 12 | 24 | 21 | 21 | 24 | 12 | 13.8 ± 0.8 | 3.8 ± 0.1 | 3.7 ± 0.1 | 15.2 ± 0.8 | 8.9 ± 2.0 |

[a]Values are group means ± SEM: litter taken as unit of calculation.

**TABLE 2.** Organ Weights in Male Rats Treated with Benomyl

| Treatment (mg/kg·d) | Body weight (g) | Testis (g) | Epididymis (g) | Seminal vesicles (g) | Prostate (g) |
|---|---|---|---|---|---|
| 0 | 584 ± 15[a] | 3.71 ± 0.06 | 1.27 ± 0.03 | 2.25 ± 0.08 | 1.20 ± 0.08 |
| 1 | 580 ± 16 | 3.59 ± 0.09 | 1.18 ± 0.04 | 2.25 ± 0.09 | 1.24 ± 0.06 |
| 5 | 573 ± 17 | 3.71 ± 0.10 | 1.27 ± 0.04 | 2.12 ± 0.12 | 1.07 ± 0.06 |
| 15 | 578 ± 18 | 3.67 ± 0.08 | 1.20 ± 0.03 | 2.18 ± 0.13 | 1.08 ± 0.05 |
| 45 | 564 ± 17 | 3.32 ± 0.08[b] | 1.07 ± 0.03[b] | 2.29 ± 0.12 | 1.10 ± 0.06 |

[a]Values are group means ± SEM.
[b]Different from control, $p < 0.05$.

effects on sperm parameters were observed at dosages of 15 mg/kg·d or less.

Histologic examination of the testis indicated a low incidence of seminiferous tubule atrophy and MNGC. The latter usually had intensely eosinophilic staining cytoplasm with pyknotic nuclei that were probably spermatidic in orgin (Fig. 1). These cells were usually free in the tubular lumens but were also observed within the tubular epithelium. The incidence of MNGC and atrophy appeared to be more prevalent in the benomyl-treated groups, but these lesions were also occasionally observed in control animals. The presence of MNGC in the testis was also often associated with their presence in the epididymis.

The percentages of seminiferous tubules containing MNGC or exhibiting obvious cell loss are given in Table 4. One animal dosed with 15 mg/kg·d had unilateral hypo- and/or aspermatogenesis affecting virtually every tubule; this animal was excluded from all data sets because the massive unilateral change was unlikely the result of benomyl treatment. Based on the number of tubules containing MNGC, the total number of abnormal tubules, and the incidence of animals showing both testicular and epididymal abnormalities, the quantitative data sug-

**TABLE 3.** Sperm Parameters in Male Rats Treated with Benomyl

| Treatment (mg/kg·d) | Motility index | Sperm concentration[b] ($\times 10^6$) | Sperm heads per testis ($\times 10^6$) | Sperm heads per gram testis ($\times 10^6$) | Sperm per cauda ($\times 10^6$) | Sperm morphology (% normal) |
|---|---|---|---|---|---|---|
| 0 | 54 ± 5[a] | 2.29 ± 0.06 | 230 ± 9 | 136 ± 4 | 229 ± 14 | 98.0 ± 0.4 |
| 1 | 58 ± 2 | 2.21 ± 0.06 | 218 ± 8 | 134 ± 3 | 221 ± 12 | 98.2 ± 0.4 |
| 5 | 46 ± 4 | 2.35 ± 0.07 | 228 ± 13 | 135 ± 7 | 224 ± 11 | 98.0 ± 0.3 |
| 15 | 48 ± 4 | 2.37 ± 0.07 | 224 ± 8 | 136 ± 5 | 211 ± 12 | 97.5 ± 0.6 |
| 45 | 52 ± 4 | 2.22 ± 0.09 | 200 ± 9 | 133 ± 4 | 130 ± 13[c] | 93.8 ± 1.1[c] |

[a]Values are group means ± SEM.
[b]Spermatozoa per milligram cauda fluid.
[c]Different from control, $p < 0.05$.

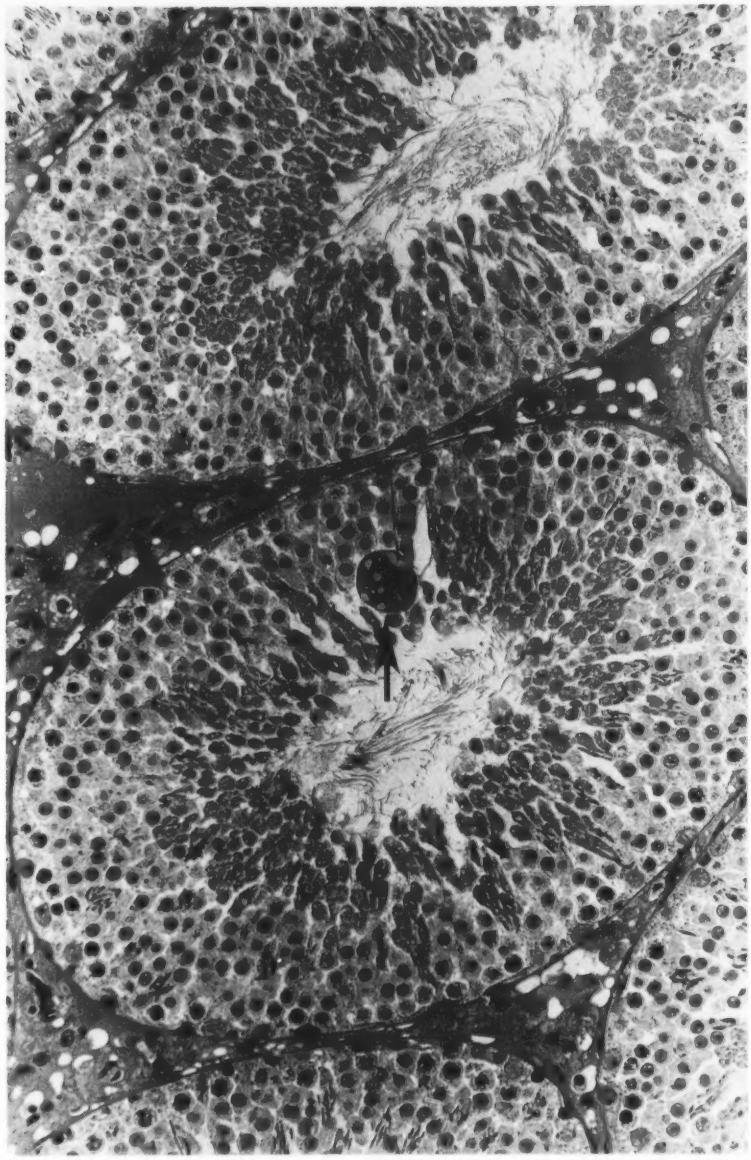

**FIGURE 1.** Cross section of seminiferous tubule from rat dosed with 45 mg/kg·d benomyl showing an MNGC (arrow).

**TABLE 4.** Incidence of Histologic Abnormalities in Male Rats Treated with Benomyl

| Treatment (mg/kg·d) | Seminiferous tubules | | | | | | | | Epididymis MNGC, animals affected |
|---|---|---|---|---|---|---|---|---|---|
| | MNGC | | Atrophy | | Partial atrophy | | Total abnormalities | | |
| | Tubules affected (%) | Animals affected | Tubules affected (%) | Animals affected | Tubules affected (%) | Animals affected | Tubules affected (%) | Animals affected | |
| 0 | 0.13 ± 0.09[a] | 3/12 | 0.02 ± 0.01 | 2/12 | 0.01 ± 0.01 | 1/12 | 0.16 ± 0.10 | 4/12 | 1/12 |
| 1 | 0.90 ± 0.87 | 6/11 | 0.19 ± 0.18 | 3/11 | 0.01 ± 0.01 | 1/11 | 1.10 ± 0.87 | 8/11 | 3/11 |
| 5 | 0.03 ± 0.01 | 3/12 | 0.04 ± 0.03 | 2/12 | 0.01 ± 0.01 | 1/12 | 0.07 ± 0.03 | 5/12 | 2/12 |
| 15 | 0.16 ± 0.07 | 5/11 | 0.11 ± 0.04[b] | 7/11[b] | 0.04 ± 0.02 | 4/11 | 0.29 ± 0.09 | 8/11 | 4/11 |
| 45 | 0.21 ± 0.08[b] | 11/12[b] | 0.05 ± 0.02 | 5/12 | 0.07 ± 0.04 | 4/12 | 0.33 ± 0.11[b] | 11/12[b] | 7/12[b] |

[a]Values are group means ± SEM.
[b]Different from control, $p < 0.05$.

**TABLE 5.** Serum Hormonal Measurements in Male Rats Treated with Benomyl

| Treatment (mg/kg·d) | LH (ng/ml) | FSH (ng/ml) | ABP (ng/ml) | Prolactin (ng/ml) |
|---|---|---|---|---|
| 0 | 0.82 ± 0.05[a] | 5.08 ± 0.33 | 62.5 ± 5.9 | 19.1 ± 3.1 |
| 1 | 0.80 ± 0.09 | 5.20 ± 0.39 | 63.6 ± 7.5 | 19.4 ± 3.8 |
| 5 | 0.80 ± 0.10 | 4.73 ± 0.40 | 67.4 ± 5.2 | 19.1 ± 2.7 |
| 15 | 0.80 ± 0.05 | 4.57 ± 0.29 | 65.7 ± 9.1 | 14.2 ± 2.8 |
| 45 | 0.79 ± 0.09 | 5.00 ± 0.25 | 58.4 ± 7.6 | 14.1 ± 2.3 |

[a]Values are group means ± SEM.

gest benomyl-related histologic changes (primarily increased MNGC) in animals dosed with 45 mg/kg·d. The percentage and incidence of tubular atrophy were increased only in animals dosed with 15 mg/kg·d; this effect therefore was considered equivocal.

Mild focal inflammation of the prostate was observed across the various groups in a few animals including one control. No changes were observed in the seminal vesicles.

The results of the serum assays are presented in Table 5. Substantial variation in prolactin values was observed across all groups, probably because of etherization of the animals. No significant changes in LH, FSH, PRL, or ABP values were detected in the benomyl-treated animals.

## DISCUSSION

Benomyl produced slight but unequivocal changes in the male reproductive system at the dosage level of 45 mg/kg·d. These changes had no detectable impact on reproductive performance, which illustrates the relative insensitivity of this end point as an indicator of other changes in the male reproductive tract. The observed no-effect level of 15 mg/kg·d is consistent with published studies in which benomyl was administered by gavage. For example, Torchinskiy et al. (1976) examined histologic and biochemical parameters in the rat testis after long-term benomyl treatment and reported decreased succinate dehydrogenase activity in testicular homogenates at a dosage level of 31.25 mg/kg·d but no change at 6.25 mg/kg·d. Although not directly comparable to effects on adult males, Kavlock et al. (1982) found slight permanent reductions in the weights of the testis and accessory sex organs in adult male offspring of dams dosed during gestation and lactation with 31.25 mg/kg·d; their no-effect level was 15.6 mg/kg·d. On a milligram per kilogram per day basis, our no-effect level differs from the dietary no-effect level of 2500 ppm reported by Sherman et al. (1975), which was based only on reproductive performance. Our no-effect level also differs from the dietary no-effect level of less than 1 ppm reported by

myl on sperm transport mechanisms, or a larger proportion of stored spermatozoa being lost via ejaculation compared to the controls.

No changes in the levels of circulating hormones were detected in the present study. The lack of change in serum LH, FSH, and PRL suggests that pituitary-hypothalamic function was not altered significantly, and that the observed testicular effects were likely due to benomyl or a metabolite acting directly on the germinal epithelium. Serum ABP concentrations were also unchanged; therefore this secretory activity of the Sertoli cell was probably not impaired. In this study, serum FSH was insensitive as an indicator of testicular damage; levels of FSH that are usually elevated in the presence of severe germ cell damage (de Kretser and Kerr, 1983) did not reflect the mild histologic changes and other antispermatogenic effects observed at 45 mg/kg·d.

The metabolic product of benomyl, MBC, binds to fungal tubulins and interferes with mitosis (Hammerschlag and Sisler, 1973; Davidse, 1973; Davidse and Flach, 1977). Although the binding affinity of MBC for mammalian tubulins appears to be less than for fungal tubulins (Ireland et al., 1979), antimitotic effects of benomyl and MBC have been shown for both in vitro and in vivo mammalian test systems (Styles and Garner, 1974; De Brabander et al., 1976). We are unaware of direct evidence that antitubulin effects are responsible for the testicular changes associated with benomyl poisoning. However, after a single oral dose of MBC to rats, Parvinen and Kormano (1974) described marked degenerations of the first and second spermatocytal divisions and also of the young step 1 spermatids. These meiotic disturbances as well as the massive sloughing of germ cells and Sertoli cell fragments observed after large oral dosages of MBC or benomyl (Parvinen and Kormano, 1974; Hess et al., 1985) resemble in some respects the responses observed after intratesticular injections of the microtubule-disruptive chemicals colchicine and vinblastine, which produce both meiotic arrest and depletion of Sertoli cell microtubules (Russel et al., 1981). In view of the potential antimicrotubule activity of benomyl, low-grade effects on tubulin-related elements of the dividing germ cells and/or the supporting Sertoli cell may have contributed to the increased MNGC and other effects observed in the present study.

In summary, repeated oral administration of 45 mg/kg·d benomyl elicited mild inhibitory effects on spermatogenesis and substantial reduction in cauda sperm reserves. No definite adverse effects were observed at 15 mg/kg·d. Decreased testis weight and decreased total sperm production appeared to be the result of germ cell damage reflected in part by increased numbers of seminiferous tubules containing MNGC. The degree of testicular damage had no detectable impact on reproductive competency and was not associated with changes in serum level of LH, FSH, PRL, and ABP. The variety of male reproductive end points used in the present study confirmed the dose-response

ranges for spermatogenic effects reported in other gavage studies of benomyl.

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